SET1B facilitates activation of the hypoxia response through site-specific histone methylation

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Abstract

Hypoxia-inducible transcription factors (HIFs) are fundamental to the cellular adaptation to low oxygen levels but how they interact with chromatin and efficiently activate their target genes is unclear. Using genome-wide mutagenesis in human cancer cells, we define genes required for HIF transcriptional activation, and identify a requirement for the Histone 3 lysine 4 (H3K4) methyltransferase SET1B. Loss of SET1B leads to a selective reduction in HIF transcriptional activity in hypoxia, with SET1B driving expression of genes involved in angiogenesis rather than glycolysis, resulting in impaired tumour establishment in SET1B deficient xenografts. Mechanistically, we show that SET1B is itself oxygen regulated, accumulates on chromatin in hypoxia, and is recruited to HIF target genes through HIF-1 α . Accordingly, we show that the hypoxic induction of H3K4me3 at specific HIF targets is both HIF and SET1B dependent, and when impaired, decreases promoter acetylation and gene expression. Together, these findings reveal SET1B as a determinant of site-specific histone methylation and provide insight into how HIF target genes are differentially regulated.

Introduction

Hypoxia inducible transcription factors (HIFs) control cellular responses to oxygen availability by activating a myriad of genes involved in angiogenesis, pH regulation, glycolysis and cell growth to promote cell survival when oxygen is scarce^{1,2}. Aside from this essential physiological response, HIFs are involved in diverse biological processes including cell development, inflammation and immune regulation, and are implicated in various disease conditions where they have been shown to promote tumour growth, alter immune responses, and modulate ischaemic damage. It is therefore paramount that we understand how HIFs are regulated as this could lead to potential new therapies.

The predominant mechanism for regulating HIFs relies on post-translational oxygen dependent degradation of the HIF- α subunit. The two main HIF- α isoforms (HIF-1 α and HIF-2 α) undergo prolyl hydroxylation by prolyl hydroxylase enzymes (PHDs) when oxygen is present, facilitating recognition and ubiquitination by the Von Hippel Lindau (VHL) E3 ligase, and subsequent proteasome-mediated degradation³⁻⁵. Degradation of HIF- α prevents the formation of an active HIF heterodimeric transcription factor with HIF1 β . In hypoxia, when oxygen levels are decreased, PHD activity is reduced, which leads to HIF- α stabilisation, dimerisation with HIF1 β , translocation of the HIF complex to the nucleus, and interaction with chromatin to activate gene transcription.

While the post-translational control of HIFs is well understood, the factors that regulate HIF activity at the level of individual target genes are less clear. In particular, how the hypoxia response is modulated across the myriad range of downstream HIF target genes remains very poorly understood. One of the primary mechanisms through which the accessibility of chromatin is controlled is by post-translational modification of histone tails. These modifications are highly dynamic and can be added or removed by families of chromatin writers and erasers. Early studies demonstrated the importance of these histone marks with the identification that p300/CBP, a lysine acetylase complex, controls HIF target gene

expression⁶. This p300/CBP HIF interaction is modulated by the oxygen-sensitive asparagine hydroxylase, Factor Inhibiting HIF (FIH)⁷⁻⁹, providing a secondary mechanism, operating in parallel with PHD activity, for turning off HIF target gene transcription in the presence of oxygen. More recently, siRNA screens in *Drosophila* and studies of human cancers identified a role for a further acetyltransferase, TIP60, along with CDK8-mediator complex as co-activators of HIF-1 α ¹⁰⁻¹². Recruitment of TIP60 enhances gene transcription of a subset of HIF-1 targets, suggesting that differential regulation of gene transcription occurs following HIF binding.

The role of histone methyltransferases in response to hypoxia is not known, but histone demethylation can be regulated in an oxygen dependent manner by lysine demethylases (KDMs). These chromatin erasers belong to the same enzymatic superfamily as the PHDs and require oxygen as a co-substrate. However, the situation is complex as recent studies demonstrate increased levels of histone 3 lysine 4 trimethylation (H3K4me3), H3K27me3 and H3K36me3 during hypoxia^{13,14}.

To date, there is no information concerning two questions. First, whether specific chromatin writer or eraser complexes can be recruited by HIF and second, whether the deposition of histone lysine modifications is dependent on the presence of the HIF complex. Here we use a flow cytometry based CRISPR/Cas9 mutagenesis screen in human cells to take an unbiased approach to identify genes needed to activate the HIF response. We identify canonical genes involved in the HIF response such as HIF1 β and HIF-2 α , but we also identify several genes that modulate chromatin marks (*UBE2A*, *PPP4C* and *SET1B*), which have previously not been implicated in the hypoxia response. The H3K4 methyltransferase SET1B (also termed *SETD1B*)¹⁵ was of particular interest as H3K4me3 marks active genes¹⁶, and its identification suggested that it may have a distinct role in hypoxia and HIF target gene activation. We demonstrate HIF-selective recruitment of this methyltransferase, resulting in site-specific H3K4 methylation and rapid activation of HIF target genes. SET1B associates with HIF-1 α through its PAS-B domain, and accumulates in the nucleus of hypoxic cells. We show that loss of SET1B decreases cell survival in cancer cells during hypoxia, and also decreases tumour establishment in xenograft models. Rather than altering expression of all HIF target genes, SET1B regulates a subset of hypoxia-induced transcripts, favouring those involved in angiogenesis without altering genes involved in glycolysis. These findings establish an ability of HIFs to selectively recruit chromatin writers to regulate gene transcription, and provide evidence that targeted H3K4 methylation by SET1B is central to tuning the metazoan response to hypoxia.

Results

Genome-wide mutagenesis screen identifies genes required to activate a HIF response.

To identify genes required to activate HIF target loci, we used a dynamic HIF-mCherry reporter^{17,18}, which reversibly accumulates in hypoxia through a fused oxygen dependent degradation domain (ODD), and requires endogenous HIF binding to a consensus triplicate HRE for activation (**Figure 1a-c**). The mutagenesis screen was performed by transducing Cas9-expressing HeLa reporter cells with a genome-

wide sgRNA library, incubating the cells in 1% oxygen for 24 hr, and undertaking iterative-flow activated cell sorting (FACS) for those cells that failed to activate the reporter (**Figure 1d, e**). sgRNAs enriched in the sorted population that failed to activate the reporter were identified by high throughput deep sequencing in comparison to sgRNAs from cells that had only been exposed to 21% oxygen (**Figure 1d, e**). HIF1b (*ARNT*) and HIF-2a (*EPAS1*) were highly enriched for sgRNA in the mutagenesis screen, validating our approach (**Figure 1e**). We also identified several genes implicated in histone modifications that were significantly enriched in hypoxic mutagenised cells compared to control cells: *SET1B*, *UBE2A* (also known as Rad6) and *PPP4C* (**Figure 1e**). *UBE2A* is a ubiquitin E2 conjugating enzyme involved in global H2B ubiquitination by the RNF20 and RNF40 E3 ligases^{19,20}. We proceeded to validate *UBE2A* by making a mixed CRISPR knockout (KO) population of HeLa reporter cells, which showed decreased activation of the HIF reporter (**Supplementary Figure 1a**). Depletion of RNF20 and RNF40 also decreased expression of the HIF reporter (**Supplementary Figure 1b**), consistent with their reported recruitment of *UBE2A* for ubiquitination^{19,20}. A role for histone acetylation in the hypoxic response was supported by the identification of *PPP4C*, a phosphatase that controls histone-deacetylation activity (*HDAC3*)²¹, which we also validated with CRISPR mixed KO populations of *PPP4C* (**Supplementary Figure 1c**).

SET1B, a mammalian H3K4 methyltransferase²², was of particular interest as it was highly enriched in the screen, and suggested that histone methylation is involved in the activation of HIF target genes. We confirmed that sgRNA depletion of *SET1B* decreased activation of the HIF reporter in hypoxia with mixed knockout populations, multiple sgRNAs and in isolated clones (**Figure 1f, Supplementary Figure 1d-f**). In all cases *SET1B* loss decreased HIF reporter fluorescent levels in hypoxia (**Figure 1f, Supplementary Figure 1d, e**), without altering HIF reporter levels in 21% oxygen (**Supplementary Figure 1g**). Although *SET1B* depletion did not prevent activation of the HIF reporter to the same extent as HIF1b loss, time course analyses showed a marked delay in reporter fluorescence with *SET1B* depletion in 1% oxygen (**Figure 1g**), consistent with the involvement of *SET1B* in activation of the HIF pathway.

The HIF transcriptional response is impaired by depletion of the *SET1B* methyltransferase.

We next examined whether *SET1B* depletion altered the activation of the endogenous HIF response, and if this was applicable to other cell types. Using the well-validated HIF-1a target, Carbonic Anhydrase 9 (CA9), we confirmed that CA9 levels decreased in HIF HeLa reporter cells, consistent with a decrease in transcriptional activation of the HIF response (**Figure 2a, b**). Similar findings were observed in other cancer cell lines (A549 lung adenocarcinoma and MCF7 breast cancer cells), and skin fibroblasts following *SET1B* sgRNA depletion in hypoxia (**Figure 2c-f**), with a similar temporal and partial decrease in CA9 levels to those observed with the HIF reporter (**Supplementary Figure 2a**). We verified that decreased activation of the reporter was not due to changes in HIF-1a levels, as *SET1B* loss did not alter HIF-1a mRNA expression or protein abundance in several cancer cell lines or immortalised skin fibroblasts (**Supplementary Figure 2b-e**). HIF-2a and HIF1b protein levels were also unchanged in *SET1B* deficient cells (**Supplementary Figure 2c-e**). Furthermore, reconstituting *SET1B* following its siRNA-mediated depletion increased CA9 levels in HeLa and A549 cells (**Figure 2g, Supplementary Figure 2f, g**), confirming the specific involvement of *SET1B* in the transcriptional activation of HIF targets.

Hypoxia activates the HIF response by decreasing prolyl hydroxylation of the HIF- α subunits, preventing their VHL dependent ubiquitination and proteasome-mediated degradation. We therefore asked whether SET1B loss in the context of PHD inhibition altered activation of HIF targets independently of hypoxia. Treating HIF reporter cells with the PHD inhibitor dimethyloxallylglycine (DMOG) stabilised HIF-1 α and activated the HIF reporter, but we observed decreased HIF reporter fluorescence levels and endogenous CA9 in SET1B depleted cells treated with DMOG (**Supplementary Figure 3a, b**). We also observed that proteasome inhibition (MG132) did not rescue CA9 levels following SET1B depletion in hypoxia (**Supplementary Figure 3e**), consistent with SET1B enhancing transcriptional activation of HIF targets rather than altering the stability of HIF-1 α or HIF targets.

Six H3K4 methyltransferases have been identified in humans: SET1A, SET1B and 4 mixed lineage leukaemia (MLL) genes, which together comprise the SET/COMPASS family²². SET1A and SET1B are closely related, each forming a large complex that shares many subunits with other COMPASS members, aside from CFP1 and WDR82²² (**Figure 2h**). Therefore, we tested if depletion of SET1A, CFP1 or WDR82 altered activation of the HIF reporter following DMOG treatment. In contrast to SET1B, SET1A depletion had no effect on HIF reporter activation (**Figure 2i, j**). However, depletion of CFP1 and WDR82, which associated with SET1B, decreased activation of the reporter similarly to SET1B loss (**Figure 2i, k, l**). Thus, the SET1B complex, but not SET1A, alters activation of the HIF reporter.

SET1B selectively drives mRNA expression of HIF target genes.

Decreased activation of the HIF-fluorescent reporter and the reduction in CA9 levels in hypoxia suggested that SET1B modulated the activation of HIF target genes. Therefore, we determined the global effect of SET1B on gene expression in hypoxia using RNA-seq. Mixed populations of SET1B knockout, HIF-1b knockout or wildtype HeLa cells were exposed to 12 hr 21% or 1% oxygen, and analysed in duplicate by RNA-seq (**Figure 3a**). SET1B depletion had a minimal effect on global gene transcription in 21% oxygen, and these changes were less than observed with HIF1b depletion alone (**Figure 3b, c**). In hypoxia, 1033 genes were significantly induced (**Supplementary Figure 4a, b**), of which 764 (74%) were decreased following HIF-1b loss (**Figure 3d, Supplementary Figure 4a, c**). SET1B loss significantly decreased the expression of 20% of these hypoxia dependent genes (**Supplementary Figure 4a, d**), favouring those that were highly upregulated in the hypoxia control HeLa cells (CA9, VEGF and PHD3) (**Figure 3d, e**). Quantitative PCR confirmed these findings in several cell types, with reduced expression of CA9, PHD3 and VEGF but not a control gene, BAP1, or the HIF-1 α target GLUT1 (**Figure 3f, Supplementary Figure 4e-g**). Moreover, reconstituting SET1B following siRNA mediated depletion of SET1B restored HIF activation of CA9 and VEGF (**Supplementary Figure 4h, i**).

Differential activation of the HIF- α isoforms did not appear to account for the subset of genes altered by SET1B loss. Analyses of available HIF-1 α and HIF-2 α binding sites from MCF7 or HepG2 cells showed no clear bias towards one isoform (**Supplementary Figure 5a-d**), although there was incomplete overlap with our RNA-seq analysis. To test HIF- α selectivity further, we generated isoform specific fluorescent reporter lines by clonal knockout of HIF-1 α , HIF-2 α or both together (**Supplementary Figure 5e, f**). HeLa HIF-1 α KO

(HIF-2 reporter) or HIF-2a KO (HIF-1 reporter) HRE-GFP^{ODD} cells, depleted of SET1B, both showed decreased GFP levels following PHD inhibition (DMOG), with combined HIF-1a/HIF-2a KO reporter cells showing no response to DMOG treatment (**Supplementary Figure 5f, g**). Thus HIF- α isoform specificity could not account for the involvement of SET1B in activation of a subset of hypoxia inducible genes.

The finding that GLUT1 was not altered by SET1B loss suggested that some cellular processes typically activated by HIFs may be differentially regulated. Gene ontology analysis supported this, as SET1B loss did not alter the transcriptional activity of all HIF target genes equally, but preferentially altered expression of genes involved in angiogenesis (**Figure 3h, Supplementary Figure 5h**), with little effect on those involved in pathways such as glycolysis (**Supplementary Figure 5h**). These findings were further supported by a marked decrease in the secretion of VEGF, a key angiogenic factor, in SET1B and HIF1b depleted A549 or HeLa cells (**Figure 3i**). In contrast, SET1B loss had a minimal effect on the HIF-dependent shift to glycolysis using bioenergetic profiling (**Supplementary Figure 6a-d**). HeLa cells treated with DMOG or selective PHD inhibitors (Roxadustat and Daprodustat) showed a shift to glycolysis, with a decrease in oxygen consumption relative to extracellular acidification (**Supplementary Figure 6a, b**). HIF1b deficient HeLa cells prevented this HIF-dependent glycolytic shift, as expected. However, SET1B deficient HeLa cells behaved the same as the wildtype cells following PHD inhibition, consistent with HIF-mediated activation of glycolysis remaining intact. Similar findings were observed in A549 cells following selective PHD inhibition, confirming that SET1B selectively regulates a subset of HIF target genes, with a strong effect on angiogenic genes and a minimal effect on glycolysis.

SET1B loss decreases cell survival in hypoxia and delays tumour establishment.

To understand the functional requirement for SET1B in the HIF response, we first examined the effect of SET1B loss on cell growth in hypoxia. HeLa or A549 mixed SET1B knockout populations were incubated in 21% or 1% oxygen and total cell numbers were measured at daily intervals. HIF1b-depleted HeLa or A549 cells were used as a control for inactivation of the HIF response. HeLa or A549 cells grew at similar rates at 21% O₂ irrespective of SET1B or HIF1b depletion (**Supplementary Figure 7a, b**), but we observed a decrease in total cell number of SET1B or HIF1b deficient cells after 24-48 hr of hypoxia (**Figure 4a, b**). These findings were consistent with the ability of the HIF response to favour cell survival in hypoxia, as demonstrated in certain cancers^{23, 24}. In these experiments, we also observed that hypoxia altered the levels of SET1B itself, with a marked transcriptional downregulation and decrease in protein levels after 24 hr in both HeLa and A549 cells (**Figure 4c-e**), suggesting that reducing SET1B expression may reduce the downstream response to HIF in prolonged hypoxia through a negative feedback loop. Importantly, the hypoxic-specific growth defect in the SET1B deficient cells was distinct from SET1A loss, which reduced cell numbers even when incubated in 21% oxygen (**Supplementary Figure 7c**), indicating a more global involvement in transcriptional regulation for SET1A. This was also evidenced by decreased mRNA of both HIF target genes and control genes following SET1A depletion (**Supplementary Figure 7d**).

We next examined whether the decrease in cell numbers in the SET1B depleted cells was due to decreased proliferation or increased cell death. Cell tracing with CFSE staining showed that proliferation

rates were not altered in HIF1b or SET1B-deficient cells irrespective of oxygen availability (**Supplementary Figure 7e**); however, there was an increase in PARP1 cleavage and caspase 3 activity in both the HIF1b and SET1B-depleted cells (**Supplementary Figure 7f, g**), consistent with increased apoptosis during hypoxia.

The contribution of HIF to cell survival in hypoxia does not only relate to growth or apoptosis, but involves activation of several pathways to improve oxygenation of tissues²⁵. Microenvironmental activation of HIF can increase vascularisation and growth, and this angiogenic effect of HIF is evident in tumour xenograft models²⁶⁻²⁸. Given the preponderance of genes influenced by SET1B involved in angiogenesis, we explored whether SET1B loss altered tumour establishment and growth using cervical (HeLa) and lung adenocarcinoma (A549) xenografts. HeLa cells or A549 cells, depleted of SET1B or HIF1b, were injected into the backs of nude mice, and the time to tumour establishment (200mm³ volume) or to reach a tumour volume of 1000mm³ was measured. In the HeLa xenografts, HIF1b or SET1B depletion resulted in a delay in tumour establishment, but not in the subsequent growth of the tumour (**Figure 4g, Supplementary Figure 7h**). A549 SET1B-deficient tumours also showed a marked delay in the initial tumour growth, which was strikingly more than observed with HIF1b loss (**Figure 4h, Supplementary Figure 7i**). Further analysis of the tumour xenografts confirmed that SET1B deficient tumours had increased hypoxic regions compared to the control A549 tumours, similarly to HIF1b loss (**Figure 4i, j**), consistent with decreased angiogenesis. Moreover, using CD31 as a marker of blood vessels within the tumour sections, we found that SET1B-deficient tumours had decreased blood vessel density (**Figure 4i, k**). Together, these findings indicate that SET1B loss alters HIF activation of angiogenic pathways, contributing to delayed tumour establishment in xenograft models.

SET1B accumulates on chromatin in hypoxia and is selectively recruited by HIF-1a.

To understand how SET1B was mechanistically involved in activating HIF target genes, we first examined the interaction of SET1B with the HIF heterodimer. Endogenous SET1B associated with both HIF-a isoforms and HIF1b (**Figure 5a, Supplementary Figure 8a-c**), however the association between HIF-1a and SET1B was more prominent. The formation of the mature heterodimeric HIF complex was not required, as SET1B still associated with HIF-1a in HIF1b null cells, indicating that the interaction was not mediated through HIF1b (**Figure 5a**). Interestingly, the interaction still occurred when HIF-1a was prolyl-hydroxylated (**Supplementary Figure 8d, e**) and was independent of HIF chromatin binding, as treatment with DNase did not prevent the association of HIF-1a with SET1B (**Figure 5b**). Other components of the SET1B complex were included in this interaction, as we observed association of HIF-1a with CFP1 (**Supplementary Figure 8f**). To map the interaction domain of HIF-1a with the SET1B complex, we generated HIF-1a truncation mutants in HEK293T cells (**Figure 5c**). Immunoprecipitated SET1B associated with HA-tagged HIF-1a constructs containing the PAS domains, but did not require the presence of the ODD or the c-terminal transactivating domain. However, a truncation mutant lacking the second PAS-B domain, a region involved in heterodimerisation with HIF-b, prevented the association with the SET1B complex (**Figure 5d, e**). Thus, recruitment of SET1B is mediated through the PAS domains on HIF-1a, and mainly through PAS-B.

SET1B has been observed to localise to the cytosol and nucleus in 21% oxygen²⁹, suggesting that its differential localisation may affect its function. We therefore determined if hypoxia altered SET1B localisation, and if HIF-1a recruited SET1B to chromatin. Subcellular fractionation studies in HeLa and A549 cells showed that SET1B was evenly distributed between the cytosol, nucleoplasm and chromatin fractions in 21% oxygen, as previously reported²⁹, but following incubation in hypoxia, increased levels of SET1B were found in the chromatin fraction (**Supplementary Figure 8g, h**). Time course analyses in HeLa or A549 cells showed an increase in the SET1B chromatin fraction after 4 hr hypoxia, with nearly all SET1B present on chromatin after 24 hr (**Figure 5f, g** **Supplementary Figure 8i, j**). This translocation of SET1B closely coincided with the movement of HIF-1a to the nucleus (**Figure 5f, g, Supplementary Figure 9i, j**). Furthermore, stabilisation of HIF-1a with DMOG also resulted in accumulation of SET1B on chromatin (**Figure 5h**), indicating that HIF stabilisation, irrespective of hypoxia, resulted in the translocation of SET1B.

To further understand the recruitment of SET1B to HREs we used ChIP-PCR at validated HIF target genes (CA9, PHD3 and VEGF). In all cases ChIP-PCR for HIF-1a confirmed that SET1B was only recruited to HIF target sites following binding of the HIF complex (**Figure 6a, b**), and that SET1B or the CFP1 component of the SET1B complex did not bind selected HIF-1 target loci in HIF1b null cells (**Figure 6c, d**). Thus, SET1B associates first with HIF-1a and is then recruited to selected HIF target genes in hypoxia.

Accumulation of H3K4me3 at HIF target genes in hypoxia is HIF dependent and mediated by SET1B.

We next determined whether SET1B altered H3K4me3 in hypoxia, and if this occurred at HIF target gene loci. HeLa or A549 cells exposed to hypoxia showed a transient increase in total levels of H3K4me3 (**Supplementary Figure 9a, b**), as previously reported, and attributed to hypoxic inhibition of lysine demethylase activity^{13, 14}. ChIP-PCR for H3K4me3 at selected HIF targets also confirmed that this mark increased in hypoxia at promoter regions (**Figure 7a**). However, H3K4me3 deposition was clearly HIF dependent, as we observed a substantial decrease in H3K4me3 levels at CA9, PHD3 and VEGF promoter regions in HIF1b null cells (**Figure 7b**). This indicated that HIF binding was associated with active deposition of H3K4me3, rather than hypoxic inhibition of demethylases. To determine if SET1B was responsible for H3K4me3 at HIF targets, we depleted SET1B in HeLa cells and used ChIP-PCR to measure H3K4me3 at HIF loci promoter regions (**Figure 7b**). SET1B depletion markedly decreased H3K4me3 at these HIF target loci in hypoxia, without altering H3K4me3 levels at the promoter of the non-HIF target BAP1 (**Figure 7b**).

To further explore the association of SET1B with H3K4 methylation in hypoxia, we used ChIP-seq to determine locus-specific changes in H3K4me3 levels across the genome in control, HIF1b or SET1B deficient HeLa cells incubated in 21% or 1% oxygen for 6 hr. In each dataset, the overall distribution of H3K4me3 was similar (**Figure 7c**) being predominantly located at the promoter regions of active genes as previously described³⁰. We then focused on those sites at which H3K4me3 was induced by hypoxia (false discovery rate - $\text{fdr} < 0.00001$) in control cells. The observed hypoxia-associated increase in H3K4me3 was reduced in both HIF1b and SET1B deficient cells (**Figure 7d**). The effect of SET1B depletion was even

more marked when H3K4me3 signal was examined at the subset of hypoxia-dependent sites that were regulated by HIF (i.e. also downregulated by HIF1b depletion in hypoxia, $\text{fdr} < 0.00001$) (**Figure 7e**). (**Figure 7e**). Analysis of individual HIF target genes that showed a transcriptional dependence on SET1B clearly demonstrated decreased deposition of H3K4me3 at promoter regions following SET1B or HIF1b loss (**Figure 7f**). The specificity for HIF target genes involved in angiogenesis was again apparent (**Supplementary Figure 9d**), with little change in H3K4me3 in several key glycolytic genes (**Supplementary Figure 9e**) or a control gene, BAP1, following SET1B loss. (**Supplementary Figure 9f**).

Lastly, we explored the relationship between H3K4me3 and acetylation as several studies have highlighted crosstalk between promoter methylation and acetylation in the control of gene transcription³¹⁻³³. We therefore measured the levels of H3K27ac at HIF target gene promoter regions by ChIP-PCR, with or without SET1B depletion (**Figure 7g**). H3K27ac increased at selected HIF target genes in hypoxia, consistent with active gene transcription³⁴ (**Figure 7g**). However, either HIF1b or SET1B depletion prevented H3K27ac deposition at the promoter regions (**Figure 7g**), providing further evidence for the recruitment of SET1B in promoting active gene transcription in hypoxia. Together, these findings demonstrate that histone methylation of a subset of HIF target loci is mediated by SET1B, and that HIFs direct this epigenetic regulation through recruitment of the SET1B complex, shedding insights into selective transcriptional regulation of the HIF response (**Figure 8**).

Discussion

This study identifies the selective recruitment of SET1B by HIFs to facilitate transcriptional activation of HIF target genes in hypoxia. Our approach of combining genome-wide mutagenesis with a dynamic HIF fluorescent reporter allowed us to dissect the genetic determinants involved in HIF activation. The identification of canonical HIF complex members (HIF-1b/ARNT and HIF-2a/EPAS1) validated our approach, as did the identification of UBE2A (Rad6), an E2 ubiquitin conjugating enzyme involved in histone ubiquitination³⁵. Depletion of the E3 ligases involved in this ubiquitination (RNF20 and RNF40) further confirmed this finding and was consistent with a role for histone 2B (H2B) monoubiquitination in gene transcription³⁶. Furthermore, increasing H2B monoubiquitination is associated with increased H3K4me3³⁷, suggesting that this step may be upstream of SET1B recruitment by HIFs. While the identification of PPP4C implicated the involvement of histone acetylation in the HIF response, via HDAC3²¹, other genes involved in histone acetylation were not significantly enriched in the screen. This may in part be due to the methodology of the screen, whereby cells underwent reoxygenation to allow cell growth between the iterative rounds of selection, and loss of some genes involved in HIF activation may have led to cell death, potentially also explaining why HIF-1a was not enriched. Alternatively, it is possible that other histone acetyltransferases may compensate for each other in the screening conditions used, as CBP/P300⁶, TIP60¹⁰ and ZMYND8³⁸ are all involved in activation of HIF target genes.

The enrichment of SET1B in the screen suggested the selective involvement of this histone methyltransferase in the HIF response. Loss of SET1B led to a reduced and delayed HIF transcriptional

response in hypoxia. This effect was specific to SET1B, as we did observe a requirement for other components of SET1 complex/COMPASS, but not the closely-related SET1A. Functional distinction between SET1 genes is consistent with prior studies demonstrating that both SET1A and SET1B are embryonic lethal at different stages of development³⁹, and that loss of either SET1B or SET1A cannot be rescued by the other⁴⁰. We found that SET1B has little involvement in basal transcription in mammalian cells, and that SET1B depletion does not alter cell growth in 21% oxygen, unlike loss of SET1A. In hypoxia, the normal diffuse subcellular localisation of SET1B²⁹ alters, with SET1B accumulating on chromatin, with kinetics similar to the accumulation of HIF-1 α on chromatin. In addition, we find that SET1B levels are transcriptionally regulated by hypoxia, providing a potentially elegant feedback mechanism to modulate activation of HIF target genes. Our data is consistent with SET1B being directly recruited to chromatin by HIFs, as we observed similar findings with DMOG treatment, and we find that both HIF- α isoforms interact with SET1B independently of DNA.

In contrast to HIF1 β loss, which completely ablates the expression of HIF target genes, SET1B loss primarily affected genes involved in angiogenesis with little effect on genes involved in glycolysis. The basis for this selectivity is unlikely to be due to HIF- α isoforms, as our HIF-1 α and HIF-2 α reporter lines demonstrate that SET1B modulates both HIF-1 and HIF-2 activity. Moreover, although HIF-2 specificity may vary between cell types, we find that several validated HIF-2 target genes (PLIN2, VEGF, and NDRG1)⁴¹⁻⁴³ are dependent on SET1B. Selective activation of HIF targets has been observed with other transcriptional coactivators such as CDK8, TIP60 and p300^{6, 10, 12, 44}, although in contrast to SET1B, CDK8 and TIP60 have a preference for genes involved in glycolysis. The mechanism for such selectivity is not clear, but postulated to be related to local recruitment of coactivators allowing a layered and nuanced response to hypoxia. It is likely that the basis for SET1B recruitment may be similar, as while changes in site-specific methylation of HIF target genes following SET1B loss are evident, H3K4me3 levels vary in amplitude and extent across promoter regions (**Figure 5f, Supplementary Figure 9d**), indicating that total levels of H3K4me3 at HIF loci cannot be the full explanation of our findings. An alternative hypothesis is that SET1B is recruited by HIFs to boost the expression of genes that are not expressed, or only expressed at low levels at 21% oxygen. For instance, CA9 is expressed at very low levels at 21% oxygen and shows a clear requirement for SET1B. However, HIF target genes that are not regulated by SET1B, such as GLUT1 and PGK, are already highly expressed in cells. There may also be some contribution by SET1A at these non-SET1B targets, as suggested by SET1A depletion decreasing GLUT1 expression (**Supplementary Figure 7d**). Lastly, non-histone methylation by SET1B may be involved. SET1A can control the transcriptional activity of YAP through its monomethylation⁴⁵, and while beyond the scope of this study, it will be of future interest to explore if SET1B methylates HIF or associated cofactors directly.

We demonstrate that SET1B-dependent deposition of H3K4me3 is directed by the presence of the HIF complex, and strikingly we observe a complete or near absence of H3K4me3 at HIF targets when HIF-1 β is depleted (**Figure 7d-f**). Several recent studies demonstrate that hypoxic inhibition of KDMs increases activating or repressing histone methylation marks^{13, 14}, and in the case of H3K4me3, this is dependent

on KDM5A activity. We now show that in addition to hypoxic regulation of KDMs, H3K4me3 deposition is directed by HIF in a predominantly SET1B dependent manner. Therefore, there is likely to be a synergistic relationship between SET1B mediated methylation and hypoxic inhibition of KDM5A, which may allow for the recruitment of the HIF complex and rapid activation of HIF target genes.

While the involvement of SET1B in activation of HIF target genes is clear, there remains active debate about whether H3K4me3 is a cause or consequence of active transcription. Our data supports a role for active transcription, as loss of SET1B leads to reduced methylation and also a reduction in HIF target gene transcription, even though the HIF complex remained bound at promoter regions (**Figure 6a, b**). Furthermore, there is also a reduction in the activating acetylation mark H3K27ac following SET1B loss and decreased H3K4me3 deposition⁴⁶. This interdependence between H3K4me3 and acetylation is consistent with other transcription factors, where the binding of a SET1A complex to p53 leads to methylation-dependent recruitment of p300³¹. Several acetyltransferases are involved in activation of HIF target genes, including CBP/P300⁶, TIP60¹⁰ and ZMYND8³⁸, and it is likely that SET1B may interact with these enzymes, which will be an important area of further study.

A primary purpose for the cellular response to hypoxia is to promote cellular survival when oxygen becomes limiting and to restore oxygen homeostasis. We demonstrate that loss of SET1B leads to a reduction in cell growth and cell survival in hypoxia in cell line models consistent with defects in the HIF pathway. Moreover, in mouse xenograft studies, SET1B loss leads to reduced tumour establishment, increased tumour hypoxia and reduced blood vessel density, similarly to the loss of HIF1 β and consistent with decreased angiogenesis. This role of SET1B in tumour growth may offer a novel therapeutic approach to modulating the HIF response. HIF-2a inhibitors, which block the dimerization of the HIF-2a PAS-B domain with HIF1b^{47, 48} are showing promising results in clear cell renal cell carcinoma⁴⁹. Targeting the interaction of SET1B with the PAS-B domain may be an adjunct to current strategies, and will be an important area to explore, particularly as acquired resistance to HIF-2a inhibitors is being observed⁵⁰. Furthermore, our findings highlight that targeting SET1B would allow a novel approach to switch off specific arms of the HIF pathway, which would be of benefit in angiogenic tumours but would not alter other important metabolic consequences of HIF activation, such as those observed in inflammatory responses.

Materials And Methods

Cell culture and reagents

HeLa, HEK293T, MCF7 and A549 cells were maintained in Dulbecco's Modified Eagles Medium (DMEM, Sigma). Skin fibroblasts cells were a gift from the Larrieu lab (CIMR) and were similarly maintained in DMEM. Hypoxic cell culture was performed in a Whitley H35 Hypoxystation (Don Whitley Scientific) at 37°C, 5% CO₂, 1% O₂ and 94% N₂. All cells were confirmed to be mycoplasma negative (Lonza, MycoAlert). All cell lines were authenticated by STR profiling (Eurofins Genomics). Full details of reagents and antibodies used as shown in **Supplementary Table 1**.

Plasmids

The following plasmids were used. pKLV-U6gRNA-EF(BbsI)-PGKpuro2ABFP (Addgene plasmid #62348), LentiCRISPR v2 (sgRNA/Cas9, F. Zhang Addgene #52961), HIF Plasmid, pHRSIN-pSFFV-HA-pPGK-Puro, pMD.G (Lentiviral VSVG), pMD.GagPol (Lentiviral Gag/Pol). Lentiviral plasmids used in the pHRSIN backbone⁵¹. The HIF1 α -^{ODD}GFP and HIF1 α -^{ODD}mCherry reporters were generated as previously described^{17,18}. HIF constructs were generated from pCDNA3 HIF-1 α (a gift from Patrick Maxwell) and cloned into the pHRSIN pSFFV backbone with puromycin resistance using NEBuilder HiFi (NEB). The SET1B construct was a gift from David Skalniak lab. SET1B was cloned into pHRSIN pSFFV backbone with puromycin resistance using NEBuilder HiFi (NEB). Primers for HIF-1 α and SET1B cloning are shown in **Supplementary Table 1**.

Lentiviral production and transduction

Lentivirus was produced by triple-transfection of HEK293T cells with the appropriate lentiviral transgene vector and the packaging vectors pCMVR8.91 (gag/pol) and pMD.G (VSVG). Transfection was performed using Trans-IT 293 reagent (Mirus) or Fugene (Promega) with cells at 70-80% confluency in 6-well plates. Viral supernatant was harvested at 48 hr, filtered through a 0.45 μ m filter, and stored at -80°C. For transduction, cells were seeded on 24-well plates in 500 μ L culture medium. 500 μ L viral supernatant was added to each well and plates centrifuged at 1800 rpm, 37°C for 1 hr. Cells were expanded and antibiotic selection was applied from 48 hr, if required.

Flow cytometry

2x10⁵ cells per sample were washed in ice-cold PBS in 5 mL round-bottom polystyrene tubes and resuspended in 500 μ L PBS/formaldehyde prior to analysis on a FACScalibur (GFP, AF488, AF647) or BD Fortessa (GFP, mCherry). For cell-surface staining, cells were washed in ice-cold PBS, resuspended and incubated at 4° for 30 min with the primary antibody. Samples were then washed with PBS and incubated with the appropriate secondary antibody at 4° for 30 min.

Whole genome CRISPR-Cas9 forward genetic screen

The Toronto human knockout library (TKO) Version 1 (Addgene 1000000069)⁵² was a gift from Jason Moffat. HeLa HRE-^{ODD}mCherry cells were transduced with Streptococcus pyogenes Cas9 (pHRSIN-FLAG-NLS-Cas9-NLS-pGK-Hygro) and selected for Cas9 expression using hygromycin. 5x10⁸ HeLa HRE-^{ODD}mCherry cells were transduced with the appropriate volume of pooled sgRNA virus (multiplicity of infection (MOI) of approximately 0.3), maintaining at least 150-fold sgRNA coverage. After 30 hr, cells were treated with puromycin 1 mg/ml for 5 days. Representation was maintained throughout the screen such that no selection event occurred where the library was cultured at fewer than 200 times the number of sgRNA sequences in the library. The library was pooled before any selection event. Fluorescence-activated cell sorting (FACS) was performed by harvesting 10⁸ cells after 24 hours of 1% O₂, washing the

cells in PBS, and then resuspending them in PBS containing 2% fetal calf serum and 10 mM HEPES (Sigma H0887). Cells were kept on ice through this process to maintain stability of the reporter. Cells were sorted using an Influx cell sorter (BD); mCherry-^{Low} cells were chosen in a gate set at one log₁₀ unit below the mode of the 1% O₂ treated control population. Cells were grown in culture and then harvested for DNA extraction. Genomic DNA was extracted using a Gentra Puregene Core kit (Qiagen). Lentiviral sgRNA inserts were amplified in a two-step PCR (with Illumina adapters added on the second PCR), as previously described⁵³. For the TKO screen, the forward inner PCR and sequencing primers were modified (**Supplementary Table 1**). Sequencing analysis was performed by first extracting the raw sequencing reads, trimming the first 20 bp, and aligning against the appropriate sgRNA library using Bowtie40. Read counts for each sgRNA were compared between conditions, and Benjamini-Hochberg false discovery rates for each gene calculated, using MAGeCK41 (**Supplementary Dataset 1**). The analysis presented compares DNA extracted following the second sort to an unsorted DNA library taken at the same time point.

CRISPR-Cas9 targeted deletions

Gene-specific CRISPR sgRNA sequences were taken from the TKO library or were designed using the E-CRISP tool were used to generate sense and antisense oligonucleotides, with 5'CACC and 3'CAAA overhangs respectively. These sgRNAs were ligated into the LentiCRISPRv2 or pKLV-U6gRNA(BbsI)-PGKpuro2ABFP vector according to published methods. CRISPR lentiviral production and transduction was performed as described. Transduced cells were selected by puromycin treatment. CRISPR-transduced cells were generally cultured for 9-10 days prior to subsequent experiments to allow sufficient times for depletion of the target protein. KO clones were isolated from the sgRNA-targeted populations by serial dilution or FACS sorted cloning and immunoblot. The sgRNAs used are shown in **Supplementary Table 1**.

Immunoblotting

Cells were lysed in an SDS lysis buffer (1% SDS, 50 mM Tris (pH 7.4), 150 mM NaCl, 10% glycerol and 5 mL/mL Benzonase nuclease) for 10 mins before heating at 70°C for 5 min. Proteins were separated by SDS-PAGE, transferred to PVDF membranes, probed with appropriate primary and secondary antibodies, and developed using ECL or Supersignal West Pico Plus Chemiluminescent (Thermo Scientific).

qPCR

Total RNA was extracted using the RNeasy Plus minikit (QIAGEN) following the manufacturer's instructions and then reversed transcribed using Protoscript II Reverse Transcriptase (NEB)). PCR was performed in ABI 7900HT Real-Time PCR system (Applied Biotechnology or Quantstudio 7 (Thermo Scientific)). Reactions were performed with 20ng of template cDNA. Transcript levels of genes were normalized to a reference index of housekeeping gene (Actin). The primers used are shown in **Supplementary Table 1**.

RNA-Seq analysis

Total RNA was extracted from HeLa cells using the RNeasy Plus minikit (QIAGEN). RNA was then used for library preparation before being sequenced on HiSeq (Illumina) by Genewiz. Analysis was undertaken by Genewiz and by our laboratory. After investigating the quality of the raw data, sequence reads were trimmed to remove possible adapter sequences and nucleotides with poor quality using Trimmomatic v.0.36. The trimmed reads were mapped to the Homo sapiens reference genome available on ENSEMBL using the STAR aligner v.2.5.2b. The STAR aligner uses a splice aligner that detects splice junctions and incorporates them to help align the entire read sequences. BAM files were generated as a result of this step. Unique gene hit counts were calculated by using feature Counts from the Subread package v.1.5.2. Only unique reads that fell within exon regions were counted. Since a strand-specific library preparation was performed, the reads were strand-specifically counted. After extraction of gene hit counts, the gene hit counts table was used for downstream differential expression analysis. Using DESeq2, a comparison of gene expression between the groups of samples was performed. The Wald test was used to generate p-values and Log2 fold changes. Genes with adjusted p-values < 0.05 and absolute log2 fold changes > 0.5 were called as differentially expressed genes for each comparison. A gene ontology analysis was performed on the statistically significant set of genes by implementing the software GeneSCF. The mgi GO list was used to cluster the set of genes based on their biological process and determine their statistical significance.

Immunoprecipitation

HeLa cells were lysed in 1% Triton TBS supplemented with 1x Roche cOmplete EDTA-free protease inhibitor cocktail for 30 min at 4°C before centrifugation at 14,000 rpm for 10 min. The supernatants were then diluted to 0.1% detergent and then pre-cleared with magnetic beads for 2 hrs at 4°C. The pre-cleared supernatant was incubated in selected primary antibody overnight with rotation at 4°C. Magnetic beads were then added for 2 hr before being washed and then bound proteins were eluted in using 2x SDS loading buffer. The eluted proteins were then separated by SDS-PAGE and immunoblotted as described.

Subcellular fractionation

1×10^6 HeLa or A549 cells were washed in PBS and then lysed in Buffer A (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and an EDTA-free protease inhibitor cocktail tablet ; Roche) and incubated with rotation at 4°C for 10 min. The supernatant containing the cytosolic fraction was collected by centrifugation at 1,400g for 4 min at 4°C. To further separate nucleoplasmic and chromatin fractions, the nuclear pellet was resuspended in Buffer B (20 mM HEPES, 1.5 mM $MgCl_2$, 300 mM NaCl, 0.5 mM DTT, 25% glycerol, 0.2 mM EDTA and an EDTA-free protease inhibitor cocktail tablet) for 10 min on ice. Sample was then centrifuged at 1,700g for 4 min at 4°C. The supernatant contains the soluble nucleoplasm and the insoluble chromatin fraction. This pellet was solubilized in 2 x SDS loading buffer containing 1:500 benzonase.

VEGF ELISA

Cells were plated on six-well plates before being treated with 1% or 21% O₂ for 24 hr. After treatment cell culture supernatant was collected and centrifuged at 1,500 rpm for 10 min at 4°C. VEGF ELISA was then performed using the Human VEGF Quantikine ELISA kit (R&D Systems) according to the manufacturer's instructions.

Bioenergetic Analyses

Extracellular acidification rates (ECAR) and oxygen consumption rate (OCR) of cells were measured as indicators of glycolysis and oxidative phosphorylation respectively, using a Seahorse XF analyzer (Agilent Technologies, Santa Clara, USA). The Glycolytic Rate Assay was performed according to the manufacturer's instructions using Seahorse XF FluxPak consumables (Agilent Technologies). Briefly, HeLa or A549 cells were depleted of HIF1 β or SET1B by sgRNA as described. Cells were plated in FluxPak 96 well plates in DMEM (Sigma Aldrich) plus 10% FBS at a cell density of 1 x 10⁴ (HeLa) or 2 x 10⁴ (A549) with or without 1mM DMOG (Cayman Chemical), 100 μ M Roxadustat (Cayman Chemical) or 100 μ M Daprodustat (Cayman Chemical), and incubated at 37°C for 24 hr. The media was then replaced with XF DMEM medium, pH 7.4 (Agilent Technologies) supplemented with 1 mM sodium pyruvate (Life Technologies), 2 mM L-glutamine (Life Technologies), 10 mM glucose (Sigma Aldrich) and incubated at 37°C in a non-CO₂ incubator for 1 hr before replacing with the same media prior to measuring in the analyzer. Programme settings were: mix 3 min, measure 3 min x 3; inject 0.5 μ M rotenone (Sigma Aldrich) and 0.5 μ M antimycin A (Sigma Aldrich); mix 3 min measure 3 min x3; inject 50 mM 2-deoxyglucose (Alfa Aesar); mix 3 min, measure 3 min x 5. Once the assay had finished the media was removed and the plate was stored at -80°C for a minimum of 24 hr prior to relative cell quantification using the CyQUANT Cell Proliferation Assay kit (Life Technologies) as per manufacturer's instructions. Results were normalised and analysed using WAVE version 2.6.1, and data was also exported to the Agilent Seahorse XF Glycolytic Rate Assay Report Generator. Graphs were prepared using GraphPad Prism version 8.

ChIP qPCR

HeLa cells grown on 15 cm dishes up a 2x10⁶ density. Proteins were crosslinked to chromatin by treatment with formaldehyde to a final concentration of 1% for 10 min. This reaction was quenched through the addition of glycine at 0.125 M for 10 min at room temperature. Cells were then washed in ice cold PBS twice and then scraped in tubes. Samples were spun at 800 g for 10 min and the supernatant was aspirated. Cells were harvested in 500mL of ChIP lysis buffer (50 mM Tris-HCl (pH 8.1), 1% SDS, 10 mM EDTA and 1 Complete Mini EDTA-free protease inhibitors (Roche). Samples were incubated on ice for 10 min and then diluted in ChIP 1:1 with ChIP dilution buffer (20 mM Tris-HCl (pH 8.1), 1% (v/v) Triton X-100, 2 mM EDTA and 150 mM NaCl). Samples were then sonicated in tubes and beads for 20 cycles of 15s on and 30s off in a Biorupter (Diagenode), followed by centrifugation for 10 min at 13,000 rpm at 4°C. The supernatants were collected. 20 mL of sample was taken at this point and stored at -20°C as an input. 200 ml of sample was diluted in dilution buffer and then preclear using 25 mL magnetic beads on a rotating wheel at 4°C for 2 hr. Immunoprecipitation was performed by incubating 1 mL of sample with antibodies overnight on a rotator. 25 ml of magnetic beads were added and incubated for 2 hr at 4°C to

capture the complexes. These were then washed sequentially and rotated for 5 min each with Wash Buffer 1 (20 mM Tris-HCl (pH 8.1), 0.1% (w/v) SDS, 1% (v/v) Triton X-100, 2 mM EDTA and 150 mM NaCl), Wash Buffer 2 (20 mM Tris-HCl (pH 8.1), 0.1% (w/v) SDS, 1% (v/v) Triton X100, 2 mM EDTA and 500 mM NaCl), and Wash Buffer 3 (10 mM Tris-HCl (pH 8.1), 0.25 M LiCl, 7 1% (v/v) NP-40, 1% (w/v) Na-deoxycholate and 1 mM EDTA), 2x with TE buffer (10 mM TrisHCl (pH 8.0) and 1 mM EDTA) and eluted with 120 µL of Elution Buffer (1% (w/v) SDS and 0.1 M Na-bicarbonate). The crosslinks were then reversed by incubating the samples and inputs with 0.2 M NaCl overnight on a thermocycler at 300 rpm at 65°C. Protein was digested using 20 mg proteinase K (Thermo Scientific) for 4 hr at 45°C. RNase H (Thermo Scientific) was added was 30 min at 37°C and then DNA was then purified using the DNA minielute kit (QIAGEN) according to the manufacturer's instructions. DNA was then used for qPCR analysis. ChIP-qPCR results are expressed relative to input material. The primers used are shown in **Supplementary Table 1**.

ChIP-seq and data analysis

ChIP was performed according to the protocol described, but upscaled by five-fold. The beads and antibody were 2x. Experiments were performed in duplicate and sample quality control and fragmentation was measured using the Bioanalyzer (Agilent). 10 ng of each ChIP sample and corresponding input were used in single end DNA library construction and 75-bp paired-end sequencing was performed using the HiSeq 4000 platform (Illumina). ChIP-seq data were processed as previously described⁵⁴ and mapped to the RefSeq hg19 (GRCh37) build of the human genome. Heat maps of H3K4me3 signal in relation to transcription start sites (TSS $\pm$ 5-kb), ranked according to gene expression in the RNA-seq analysis, and line graphs showing the average (mean) signal across these regions were generated using ngsplot 2.6.1⁵⁵. Signal intensities at specific gene loci were displayed using IGV Genome Browser⁵⁶ v 2.7.2.

ChIP-seq peaks were identified using both the T-PIC (Tree shape Peak Identification for ChIP-Seq)⁵⁷ and MACS (Model-based analysis of ChIP-Seq) and only peaks identified with both peak callers were considered. Differentially bound peaks ($\text{fdr} < 10^{-5}$) were identified using DiffBind v2.16.0⁵⁸. Overlapping peaks were identified using BEDTools v2.17.0⁵⁹ and normalised read counts for each peak were determined using samtools v0.1.19⁶⁰.

siRNA-mediated depletion

siRNA SMARTpools for SET1B (Dharmacon) or MISSION siRNA Universal Negative Control were transfected into HeLa or A549 cells using Lipofectamine RNAi MAX (Thermo Fisher) according to the manufacturer's protocols. Cells were harvested after 48 hr for further analysis by flow cytometry, qPCR, or immunoblot.

Cell proliferation and death assays

Cells were counted and plated on six well plates at a density of 5×10^4 cells for HeLa or 1×10^5 cells for A549 cells. Cells were then counted every 24 hr for three days when cells were grown either at 21% or 1% O₂. Cells were counted using a haemocytometer in triplicate. Cell division was also assessed using both CFSE assay (CellTrace CFSE Proliferation kit, Thermo Scientific). Cell death via apoptosis was measured using a caspase 3 activity kit (CellEvent Caspase 3/7, Thermo Fischer), according to manufacturer's instructions.

Tumour xenografts

HeLa and A549 cells were resuspended in chilled matrigel and PBS (mixed 1:1) and implanted subcutaneously (0.1 ml of 5×10^7 /ml) into female (HeLa) and male and female (A549) athymic nude mice Crl: NU(NCr)-Foxn1^{nu} (Charles River Laboratories, Inc), aged 8-9 weeks. Mice were randomly assigned into groups (using a randomization function =RAND(); excel) that received wildtype, HIF1 β or SET1B depleted tumour cells. Tumour length (L), width (W) and height (H) were measured with calipers, and tumour volume was calculated with the formula $(L \times W \times H) = \text{volume (mm}^3\text{)}$. Tumour establishment was defined as a palpable tumour mass of 200 mm³. Tumours were excised at maximum burden (1000 mm³) and snap frozen. Investigators were blinded to the tumour group status during tumour measurement. Two hours prior to sacrifice and tumour excision, mice received pimonidazole (60mg/kg dosed in 0.1 ml/10 g body weight, intra-peritoneal injection). All mice were housed in specific pathogen-free animal facilities (at 20-23°C, with 40–60% humidity, 12 hr light/12 hr dark cycle). Sample size was calculated using the Resource Equation. Procedures were ethically approved by the University of Cambridge Animal Welfare and Ethical Review Body (AWERB) and complied with the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012, under the authority of a UK Home Office License. The ARRIVE (Animal Research: Reporting *In Vivo* Experiments) guidelines (<https://www.nc3rs.org.uk/arriveguidelines>) were used for planning, conducting and reporting of experiments.

Immunofluorescence

Frozen tumour sections (8 μ m) were fixed in acetone at -20 °C (10 min) and blocked with 10% horse serum, PBS (15 min). Sections were incubated with primary antibodies anti-mouse CD31 (BD Pharmingen, UK, #553370) and Pimonidazole (Hydroxyprobe 1 clone 4.3.11.3, Natural Pharmacia International Inc, USA), both diluted 1:50 in a humidified container for 18 h (4°C), washed, incubated with secondary Alexafluor-labelled antibodies (1:500), washed, counterstained with 4',6-diamidino-2-phenylindole (DAPI, (5mg/ml diluted 1:5000) and mounted in fluorescent mounting media (DAKO UK Ltd, UK).

Visualisation and quantitation of tumour sections

Immuno-fluorescently labelled tumour sections were digitized on Axioimager Z2 Upright Microscope (Zeiss) using Zen Blue Pro 2012 software (version 1.1.2.0; Zeiss). For all analyses at least two

independent sections were analysed per tumour and analyses were performed using ImageJ software (version 1.52a). For quantification of pimonidazole-positive regions; a positive staining threshold was determined against background staining of tumour sections stained with secondary Alexafluor-labelled antibodies (1:500) only. Values above this threshold were deemed positive. Regions giving false positive staining (tumour edges that had lifted slightly and regions of necrotic cells, which bind secondary antibodies non-specifically) were excluded from the analyses. Hypoxic fraction was determined by analysing the area of pimonidazole positive staining within the tumour section area.

For vessel analyses, an 8x8 square grid was overlaid onto the tumour sections, six squares (area per square 0.175mm²) were randomly selected (using a randomization function =RAND(); excel) and analysis was performed within these six regions.

Tumour necrosis was identified by DAPI staining and defined as regions with fragmentation and/or disappearance of nuclear labelling as previously described⁶¹, with tumour cells visible to variable extent. Percentage tumour necrosis was determined by analysing the area of necrotic regions within the tumour section area.

Statistical Analyses

Quantification and data analysis of experiments are expressed as mean $\pm$ SD and P values were calculated using two-way ANOVA or two-tailed Student's t-test for pairwise comparisons, and were calculated using Graphpad Prism version 8. Qualitative experiments were repeated independently to confirm accuracy. Statistical analysis of the screen was performed using MAGeCK version 0.5.5⁶², testing the sgRNA read counts obtained following the second sort against sgRNA read counts obtained from unsorted cells lysed at the same time point. Statistical analyses for the RNA-seq and ChIP-seq are described in the respective sections.

Declarations

Data Availability

SgRNA read count tables from the CRISPR/Cas9 genetic screen are shown in **Supplementary Dataset 1**. RNA-seq data and ChIP-seq will be deposited at Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/>).

Author Contributions

Conceptualization, BMO and JAN; Methodology, BMO, NB, PSJB, ITL, DRM, PHM and and JAN; Investigation, BMO, NB, PSJB, BMO, ITL, AP, LR, RS DRM, and JAN; Writing – original draft, BMO and JAN; Writing – reviewing and editing, BMO, NB, DRM, PHM, PJR and JAN; Funding acquisition, JAN; Resources, DRM, PJR, PHM and JAN; Supervision, JAN.

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Declaration of Interests

Peter J Ratcliffe is a scientific co-founder of ReOx Ltd., a company, which is developing inhibitors of the HIF hydroxylase enzymes. The other authors declare no competing interests.

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Figures

Figure 1

Genome-wide mutagenesis screen identifies genes required to activate a HIF response. (a) Schematic of the HRE-ODDmCherryODD reporter construct. HeLa HRE-ODDmCherry reporter cells stably expressing Cas9 were transduced with a sgRNA against HIF1 β and treated with 21% or 1% O₂ for 24 hr. Samples were analysed using flow cytometry (b) or by immunoblotting (c) using the indicated antibodies. (d) HeLa HRE-ODDmCherry cells were transduced and mutagenized with genome-wide sgRNA library (Toronto KO library). mCherry^{LOW} cells following 24 hr at 1% O₂ were selected by iterative FACs and sgRNAs

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Figure 2

The HIF transcriptional response is impaired following SET1B loss. (a, b) Cell surface CA9 was measured and quantified in HeLa cells depleted of SET1B by sgRNA after 7-10 days, and then incubated in 21% or 1% overnight. Quantification of geometric mean of cell surface CA9 staining (b) ($n=3$, mean $\pm$ SD, *** $p < 0.0001$, two way ANOVA). (c) A549, (d) HeLa, (e) MCF7, and (f) Skin Fibroblasts cells stably expressing Cas9 were transduced with an sgRNA against HIF1 β or SET1B and after 7-10 days treated with 21% or 1% O₂ for 24 hr. Samples were analysed using indicated antibodies ($n=3$). (g) Reconstitution of SET1B siRNA depleted cells with overexpressed SET1B. HeLa cells were transfected with a control or a SET1B siRNA, with and without a SET1B overexpression plasmid for 48 hr. Cells were then incubated at 21% or 1% O₂ for a further 24 hr before lysis. Endogenous SET1B, HIF1 α and CA9 levels were detected by immunoblot. $n=3$ (h) Schematic of the SET1A and SET1B containing complexes highlighting core histone methyltransferase complex members (green) and proteins specific for SET1A and SET1B containing complexes (red). (i-k) CRISPR Cas9 depletion of SET1 complex proteins in HeLa HRE-ODDGFP Cas9 cells. Cells were transduced with sgRNA targeting SET1A, SET1B, WDR82 and CFP1. After 7-10 days cells were treated with or without 1 mM DMOG 24 hr, and activation of the HIF GFP reporter measured using flow cytometry (i). sgRNA depletion of SET1 complex proteins was measured by immunoblotting (j, k). (l) Endogenous SET1B was immunoprecipitated in HeLa cells grown at 21% or 1% O₂ for 6 hr. Samples were immunoblotted for SET1 complex members CFP1 and WDR82.

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a Control HIF1 β KO SET1B KO
21% O₂ (12 hr) 1% O₂ (12 hr)
RNA Extraction, library prep and sequencing

b HIF1 β KO vs. Ct in 21% oxygen
-log₁₀(p value)
log₂(fold change)

c SET1B KO vs. Ct in 21% oxygen
-log₁₀(p value)
log₂(fold change)

d mRNA expression in 1% oxygen
Downregulated Upregulated
-log₁₀(p value)
log₂(fold change)
● HIF1 β dependent
● SET1B dependent
● HIF1 β /SET1B dependent

e Control HIF1 β KO SET1B KO
ALDOC
ANKRD37
CA9
EGLN3
ENO2
HEY1
HMOX1
INSIG2
LOX
P4HA2
PDGFB
PLIN2
SERPINE1
SLC2A1
SLC2A3
VEGFA
log₂(fold change)

f CA9 PHD3 VEGF GLUT1 BAP1
Relative mRNA induction
1% O₂: - + - + - +
sgRNA: Ct HIF1 β SET1B

g SET1B KO HIF1 β KO Control
angiogenesis genes (GO:0001525)
ADAMTS1
ANGPT1
ANGPT2
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Significantly up or downregulated genes were selected based on a $\log_2(\text{fold change})$ of >0.5 or <-0.5 . Genes significantly upregulated are displayed in red and downregulated are displayed in blue. (d) RNA-seq HIF1 β and/or SET1B dependent mRNA expression in 1% oxygen. Volcano plot of $\log_2(\text{fold change})$ against $-\log_{10}$ p-value for all genes in 21% and 1% O₂. HIF1 β dependent genes (red), SET1B dependent genes (light green) and combined HIF1 β and SET1B dependent genes (dark green) are shown. (e) Heat map displaying $\log_2(\text{fold change})$ in mRNA expression in control, HIF1 β or SET1B mixed KO cells of validated HIF target genes. (f, g) Quantitative PCR (qPCR) of HIF targets (CA9, PHD3, VEGF and GLUT1) and a non HIF target (BAP1) in HeLa and A549 cells following HIF1 β or SET1B depletion by sgRNA after 7-10 days, and incubation in 1% or 21% oxygen for 24 hr ($n=3$, mean $\pm$ SD, *** $p < 0.0001$, two-way ANOVA). (h) Heat map comparing the $\log_2(\text{fold change})$ between control, HIF1 β and SET1B depleted cells focussing on genes involved in angiogenesis. (i) VEGF ELISA from control, HIF1 β or SET1B depleted HeLa or A549 cells. Cells were grown in 21% or 1% O₂ before supernatants were collected ($n=3$, mean $\pm$ SD, *** $p < 0.0001$, two way ANOVA).

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Figure 4

SET1B loss decreases cell survival in hypoxia and delays tumour establishment in vivo. (a, b) Cell growth in hypoxia following depletion of SET1B or HIF1 β . Proliferation assays of HeLa (a) or A549 (b) depleted of HIF1 β or SET1B grown in 21% or 1% oxygen for indicated number of days (n=3, mean, ***p < 0.001, ***p < 0.0001, two way ANOVA at 3 days). (c, d) Time course of SET1B and HIF-1 α protein levels in 1% oxygen. HeLa and A549 cells were incubated in 1% O₂ for 0 to 24 hr as indicated, lysed, and immunoblotted for

SET1B and HIF-1 α . β -actin was used as a loading control. (e) qPCR analysis of SET1B and CA9 mRNA levels in HeLa and A549 cells treated with 21% or 1% O₂ for 24 hr (n=3, mean $\pm$ SD, *p < 0.05, **p < 0.01, ***p < 0.0001). (g, h) HeLa and A549 tumour xenograft models. Mice for each condition were injected subcutaneously with control and HIF1 β or SET1B mixed KO cells (generated by sgRNA). Tumour establishment was determined by the time it took for tumours to reach 250 mm³ (n=7, mean $\pm$ SD *p < 0.05, unpaired t test). (i) Representative images of A549 tumour sections stained with antibodies against CD31 (green; to detect vessels), pimonidazole (red; to detect hypoxic regions) and DAPI (blue; binds nuclei, used to identify necrotic regions). Scale bar 500 μ m. (j) Increased tumour hypoxia in HIF1 β and SET1B depleted tumours compared to WT, (n= 6 (control and HIF1 β), 7 SET1B, mean $\pm$ SD, *p < 0.05, unpaired t test). (k) Decreased vessel density (number of vessels/mm²) in SET1B and HIF1 β depleted tumours compared to control, (n= 6 (control and HIF1 β), 7 SET1B, mean $\pm$ SD, *p < 0.05, unpaired t-test).

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Figure 5

SET1B associates with HIF-1 α and accumulates on chromatin in hypoxia (a, b) The interaction of SET1B with the HIF complex is not dependent on HIF1 β or DNA. SET1B was immunoprecipitated from wildtype and HIF1 β mixed KO HeLa cells incubated in 21% O₂ or 6 hr of 1% O₂ (a). Endogenous SET1B was immunoprecipitated from cells in 21% O₂ or 6 hr at 1% O₂, with or without DNase treatment (Benzonase, 5 μ l/mg)). Samples were analysed by immunoblotting using indicated antibodies. (c-e) Mapping the

interaction domain of HIF-1 α with SET1B. (c) Schematic of full length HIF-1 α and truncation mutants. Characterised functional domains are highlighted: basic helix-loop-helix (bHLH), Per-Arnt-Sim (PAS) domain, oxygen dependent degradation (ODD) domain, n-terminal (NAD) and c-terminal (CAD) transactivation domain. (d) Expression levels of HIF-1 α truncation mutants expressed in HEK293T cells. (e) HEK293T cells were transfected with 4 μ g of the indicated expression constructs for 48 hr prior to treatment with 1% O₂ for 6 hr. Endogenous SET1B was immunoprecipitated and immunoblotted (n=3). (f, g) Cell fractionation was performed on HeLa cells treated at 21% or 1% O₂ for indicated times. Samples were immunoblotted and the ratio of SET1B in different cellular compartments was quantified using ImageJ (g) (n=3, mean $\pm$ SD). (h) Cell fractionation was performed on HeLa cells treated with and without 1mM DMOG for 24 hr. Immunoblotting was performed using indicated antibodies (representative of n=3).

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Figure 6

HIF-1α selectively recruits SET1B to HIF target genes (a-d) Chromatin immunoprecipitation (ChIP) was performed in control, HIF1β and SET1B sgRNA depleted HeLa cells incubated in 21% or 1% O₂ for 6 hr for HIF-1α (a), HIF1β (b), SET1B (c) or CFP1 (d). ChIP qPCR was performed using primers targeting hypoxia responsive elements (HRE) within the promoters of selected HIF target genes (n=at least 3 replicates, mean ± SD *p < 0.05, **p < 0.01, ***p < 0.0001, two way ANOVA).

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Figure 7

SET1B enhances H3K4me3 at the promoter regions of HIF target genes (a) H3K4me3 increases at HIF target gene promoters in hypoxia. H3K4me3 ChIP was performed on HeLa cells incubated at 21% or 1% O₂ for 6 hr. The samples were then analysed by qPCR using primers targeting the HREs within the promoters of selected HIF target genes (CA9, PHD3 and VEGF) and a primer targeting the promoter of BAP1. (n= at least 3 replicates, mean $\pm$ SD, ***p < 0.0001, two way ANOVA) (b) Loss of the HIF complex or

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Figure 8

SET1B is recruited by HIF to facilitate activation of the HIF response. Model of recruitment of SET1B by HIFs to facilitate expression of specific HIF target genes in hypoxia. In normoxia HIF1 α is degraded via oxygen-dependent proteasomal degradation, and SET1B is predominantly present within the cytosol. In hypoxia, HIF-1 α is stabilized, dimerises with HIF1 β and interacts with SET1B. SET1B accumulates in chromatin in hypoxia, and the HIF complex directs SET1B to specific HIF target genes where it deposits

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